Pseudomonas graminis strain CPA-7 differentially modulates the oxidative response of fresh-cut 'Golden delicious' apple depending on the storage conditions.

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Abstract

Oxidative response of fresh-cut antioxidant-treated 'Golden delicious' apples during chilling storage was differentially modified by the biopreservative bacterium CPA-7 depending on the conditions; passive modified atmosphere packaging (MAP) or aerobic, the latter being more favorable for the antagonist growth. Inoculation with CPA-7 had no influence on fruit quality parameters in any of the conditions tested. During the first 24 h both in air or in MAP, ascorbate peroxidase (APX) activity triplicated the initial level in response to CPA-7 inoculation, reaching up to 4-fold the activity of non-inoculated fruit (control). From 24 h of storage in MAP, polyphenol oxidase (PPO) activity was sharply enhanced in response to CPA-7 attaining values up to 6-fold higher than that of the control at the end of storage. Contrastingly, from 1 d to 3 d of storage in MAP CPA-7 suppressed catalase (CAT) activity by 1.5-fold. Subsequently, after 3 d in such conditions, superoxide dismutase (SOD) and PPO activities were almost duplicated in the presence of CPA-7 if compared to the control. On the other hand, when stored in air, polyphenol oxidase (POX) showed a biphasic

induction in response to CPA-7 after 3 d and 6 d of incubation. At day 6, the activity of this enzyme reached values 8-fold higher in response to CPA-7 than the observed for the control regardless of storage conditions, yet it was not paired to an increase of browning. Inoculation with CPA-7 led to the slowdown of the decline of antioxidant capacity in air, which contrasted with the response upon MAP conditions. These results suggest that CPA-7 may trigger the activation of the fruit defense-response thereby mitigating its oxidative damage. Such activation may play a role as a putative biocontrol mechanism against foodborne pathogen infections.

1. Introduction

Control of foodborne diseases through bio-friendly strategies circumventing the usage of chemicals that produce potentially harmful residues in fresh-cut fruit is an important area of research. The use of antagonistic epiphytic microorganisms is a method that fulfills this characteristic which has been widely explored over the past (Belak and Maraz, 2015; Leverentz et al., 2006). In addition to the maintenance of microbiological quality, the growing demand of fresh processed fruit and vegetables has challenged food industry to also guarantee the physicochemical acceptance of these products in spite of the metabolic changes inherent to this type of commodities, mainly associated to abiotic stress caused by both refrigerated storage and processing. In this sense, cold storage in regular controlled atmosphere conditions is known to be stressful to harvested fruit inducing the accumulation of ROS (Chiriboga et al., 2013). Thus said, scarce information is available about the oxidative changes of fresh-cut fruit at the enzymatic or metabolic level (Larrigaudiere et al., 2008).

Besides, cutting of fruit flesh compromise compartmentalization in nearby cells allowing phenolic compounds located in vacuoles to get in contact with polyphenol oxidase (PPO), located in plastids, triggering the reaction known as enzymatic browning (Holderbaum et al., 2010). Other antioxidant enzymes such as peroxidase (POX) has also been linked to polyphenol-associated browning through a coordinated mechanism

55 involving the accumulation of its substrate, hydrogen peroxide (H_2O_2), stimulated by the
56 PPO-mediated generation of quinones (Jiang and Miles, 1993). Concomitantly, H_2O_2 is
57 highly oxidant and may damage membrane and other cellular components through
58 several mechanisms. Thus, in both fresh-cut and intact fruit, its removal is rapidly
59 activated in order to protect plant cells from these damages. H_2O_2- scavenging
60 mechanisms include enzymatic reactions which involve superoxide dismutase (SOD),
61 catalase (CAT) and ascorbate peroxidase (APX) found in the cytosol, chloroplasts and
62 mitochondria of higher plants (Hung et al., 2005) as well as non-enzymatic antioxidants
63 including phenolic compounds (vitamin E, flavonoids, phenolic acids and other
64 phenols); nitrogen compounds (alkaloids, amino acids and amines), carotenoids and
65 chlorophyll derivatives (Kumar, 2012).

66 The control of enzymatic browning in the fresh- cut produce industry is currently mainly
67 based on the modulation of PPO activity through antioxidant formulations that
68 commonly contain reducing compounds such as ascorbic acid and its derivatives,
69 cysteine and glutathione (Rupasinghe et al., 2005). These agents are hypothesized to
70 control PPO activity either by reducing quinones to the native diphenols or by reacting
71 irreversibly with o- quinones to form stable colourless products (Nicolas et al., 1994; He
72 and Luo, 2007).

73 In addition, plant oxidative metabolism is also activated in response to biotic stress as a
74 part of induced defense mechanisms (Pieterse et al., 2000, 2014). Under the light of
75 the increasing knowledge about plant-microorganism interactions, induced resistance
76 has arisen as a method for controlling postharvest diseases. The investigation of these
77 processes has revealed that biocontrol agents (BCA) activate defense-related enzymes
78 such as phenylalanine ammonia lyase (PAL), POX, PPO and chalcone synthase
79 among others, therefore contributing to better antioxidant defenses and tolerance to
80 pathogens attack (Chen et al., 2000; Jain et al., 2012; Alkan and Fortes, 2015).

Pseudomonas graminis strain CPA-7 is an aerobic epiphyte bacillus which has been used as BCA against foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* on fresh cut apples, melons and peaches (Abadias et al., 2014; Alegre et al., 2013a, 2013b; Collazo et al., 2017). Although attempts to elucidate the mechanisms underlying its antagonistic activity have been carried out, they still remain poorly understood (Collazo et al., 2017). Thus, we hypothesized that a putative mode of action for CPA-7 could be the activation of defense mechanisms in fresh-cut fruit leading to a better control of foodborne pathogens populations. Accordingly, the oxidative metabolism of inoculated fresh-cut apples treated with an antibrowning agent both upon aerobic conditions, suitable for CPA-7 growth, and in semi-commercial conditions (modified atmosphere packaging, MAP) were investigated.

2. Materials and methods

2.1 Fruit processing

Apples (*Malus domestica* L. cv. Golden Delicious) were obtained from local packing-houses (Lleida, Catalonia, Spain). Prior to experimental assays, the fruit were washed with running tap water, surface disinfected with 700 mL L^{-1} ethanol, peeled with an electric fruit peeler and cut into eight wedges with a handheld corer/slicer. Wedges were kept in chilled (5°C) chlorinated tap water (pH 6) until they were subsequently treated or stored.

2.2 Antagonist culture conditions

For the antagonist inoculum preparation, a single colony of *Pseudomonas graminis* strain CPA-7 grown in tryptone soy agar plates (TSA, Biokar, Beauveais, France) during 48 h at 30 ºC, was inoculated into 50 mL of tryptone soy broth (TSB, Biokar, Beauveais, France) and incubated in aerobic conditions for 20-24 h in agitation (15.71 rad s^{-1}) at 25 ºC. Bacterial cells were harvested by centrifugation at 9800 × g for 10 min at 10 °C and re-suspended in sterile distilled water. The concentration of the

suspension was checked by plate count of appropriate 10-fold dilutions in saline peptone (8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) onto TSA plates after incubation at 30 °C for 48 h.

2.3 Inoculation, sampling and microbiological analysis

Suspensions of CPA-7 at 10^7 CFU mL^{-1} were prepared in a 60 g L^{-1} NatureSeal® AS1 AgriCoat Ltd., Great Shefford, UK) antioxidant aqueous cold solution (4 ºC), as recommended by the manufacturer. Non-inoculated antioxidant solution was included as control. Fruit wedges were dipped-inoculated at a ratio 1:2 (weight of product: volume of suspension) for 2 m in agitation (15.71 rad s^{-1}) in tabletop orbital shaker Unimax 1010 (Heidolph, Germany). After drainage of the excess of water, 10 g of apple wedges were used to determine initial microbiological quality in triplicate and three samples of about 70 g were frozen with liquid nitrogen, grinded in a commercial grinder (Minimoka 6R-020, Coffeemotion, Lleida, Spain) and stored at -80 °C until biochemical analysis. The rest of the treated fresh-cut fruit was packaged (120 g per replicate) in 400 mL polyethylene terephthalate ShelfMaster™ Pronto™ trays (PlusPack, Denmark). Each tray was considered as a replicate and three replicates per treatment and sampling time were used. Trays were sealed with a peelable plastic film (polyester anti-fog film (OALF) of 14 µm of thickness + oriented polypropylene (OPP) of 20 µm of thickness) with a line of holes of 60 - 80 µm each and 75 mm separation spacing between them, to achieve passive modified atmosphere. In a parallel set of trays the film was macro perforated (nine extra holes per tray) in order to attain aerobic conditions. Trays were stored at 5 °C in darkness and biochemical and microbiological analyses were performed at 0, 1, 3 and 6 d post-inoculation. Population dynamics of CPA-7 as well as total mesofils of inoculated and non-inoculated samples were analyzed at each sampling time. For this, 10 g of apple from each tray was homogenized in 90 mL of buffered peptone water (APT, Biokar, Beauvais, France) and tested by viable cells count onto TSA and plate count agar plates (PCA, Biokar,

Beauveais, France) incubated at 30 °C (for CPA-7) and at 25 °C (for mesofils) for 48 h and 72 h, respectively.

2.4 Fruit physicochemical quality parameters

Firmness, color and pH of fresh-cut wedges were determined initially and at each sampling time as described elsewhere (Alegre et al., 2013a). Low values of CIE coordinate L* and high values of a* were considered as indicators of surface browning intensity (Sapers and Douglas, 1987). Soluble solids and titratable acidity were measured in two samples per tray initially and at the end of storage. Soluble solids concentration at 20 °C was expressed as mass fraction of sugars relative to the fruit (%). Acidity was measured in 10 mL of pulp and was expressed as malic acid content (g L^{-1} juice).

2.5 Biochemical analyses

2.5.1 Total antioxidant capacity (TAC) and total phenolic content (TPC)

Extracts for total phenolic content (TPC) and total antioxidant capacity (TAC) determination were prepared by mixing 3 g of frozen fresh pulp with 10 mL of a solution containing 19.7 mol L^{-1} methanol, 0.05 mol L^{-1} HCl. The mixture was held in agitation (20.94 rad s^{-1}) for 2 h and centrifuged at 24 000 x g at 4 ºC. TPC was quantified by measuring the optical density (OD) at 765 nm in a spectrophotometer (EONC, Biotek Instruments, Highland Park, VT, USA) after the reaction of 0.05 mL of each extract with 0.25 mL of Folin-Ciocalteu reagent and 0.5 mL of 1.9 mol L^{-1} Na_2CO_4.

TAC was determined by measuring OD at 593 nm of the above mentioned extracts following the Ferric Reducing Antioxidant Power (FRAP) protocol (Giné-Bordonaba and Terry, 2016).

2.5.2 Hydrogen peroxide production and malondialdehyde (MDA) content

Malondialdehyde (MDA) was quantified in 0.5 g of frozen fresh pulp as a marker of lipid peroxidation using the thiobarbituric acid reactive substrates (TBARS) assay described

by Martínez-Solano et al. (2005). The OD of the supernatants after reaction was measured at 532 nm and substracted to the inespecific absorption read at 600 nm. Quantification of TBARS was calculated by its extinction coefficient of 155 mmol L^{-1} cm $^{-1}$.

To determine hydrogen peroxide levels, 5 g of frozen fresh pulp was homogenized in 7.5 mL of 0.5 mol L^{-1} trichloroacetic acid, filtered through two layers of Miracloth (Textil Planas Oliverassa, Manresa, Spain) and centrifuged at 20 000 x g for 15 min at 4 ºC. H_2O_2 content was determined using the aqueous peroxide colorimetric assay, PeroxiDetect™ Kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions. This procedure is based on the measurement of the color change at 560 nm of adduct formed by Fe^{3+} ion and xylenol orange (XO, PubChem CID 73041), during the oxidation by peroxide of Fe^{2+} to Fe^{3+} ions at acidic pH. The molar extinction coefficient of the XO-Fe^{3+} colored adduct in aqueous solution at 560 nm is 15 000.

2.5.3 Activities of enzymes involved in antioxidant metabolism

2.5.2.1 Peroxidase (POX) y Polyphenol oxidase (PPO)

Total peroxidase (POX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.14.18.1) were extracted from 10 g of frozen fresh pulp as described by Giné-Bordonaba et al. (2017). POX activity was measured as the optical density at 470 nm following the reaction of the extract with 10 mmol L^{-1} guaiacol and 10 mmol L^{-1} H_2O_2, according to the method described by Lurie et al. (1997). PPO activity was determined at 400 nm in a reaction mixture containing 0.1 mol L^{-1} potassium phosphate buffer (pH 6) and 65 mmol L^{-1} pyrocatechol together with the correspondent enzyme extract as described by Vilaplana et al. (2006).

2.5.2.2 Superoxide dismutase (SOD) and catalase (CAT)

For the extraction of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), 5 g of frozen fresh pulp were homogenized in 15 mL 0.1 mol L^{-1} potassium phosphate buffer (pH 7.8), 2 mmol L^{-1} dithiothreitol (DTT), 50 g L^{-1}

polyvinylpolypyrrolidone (PVPP), 0.1 mmol L^{-1} ethylene diamino tetracetic acid (EDTA) and 1.25 mmol L^{-1} polyethylene glycol. The homogenized was filtered, centrifuged and purified as described by Giné-Bordonaba et al. (2017). In turn, SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm following the method of Gianopolitis and Ries (1977).

2.5.2.3 Ascorbate peroxidase (APX)

For ascorbate peroxidase (APX; EC 1.11.1.11) extraction, 10 g of frozen fresh pulp was homogenized with 30 mL of 0.1 mol L^{-1} base phosphate buffer (pH 7.5) containing 0.8 mmol L^{-1} HCl, 1 mmol L^{-1} EDTA, filtered through two layers of Miracloth and centrifuged at 10 000 x g for 15 min at 4 ºC. APX activity was determined at 290 nm during 10 min by monitoring the H_2O_2-dependent decomposition of ascorbate in a mixture containing twenty microliters of the recovered supernatant and 280 µL of a reaction solution (0.22 mmol L^{-1} ascorbic acid, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} H_2O_2) (Nakano and Asada, 1981).

2.5.2.4 Protein content

Protein content of all extracts was determined by the Bradford method at 595 nm using a protein assay kit (Bio-Rad, München, Germany). Protein reagent was mixed with the correspondent phosphate buffer (pH 6, 7 or 7.8) used for each enzyme extraction in a ratio 1:3.6. Bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) was used as standard.

2.6 Expression of results and statistical analysis

Enzyme activity was expressed in units of enzyme activity per milligram of protein (U mg^{-1} protein). For SOD, 1 U represents the amount of enzyme required to inhibit NBT reduction by 50 % of initial amount. For the rest of the analyzed enzymes, 1 U represents the quantity of enzyme responsible for a change in 1 absorbance unit per minute. Microbiological data was estimated as microbial counts per gram of fruit (CFU

g^{-1} fruit). Non-enzymatic antioxidant activities were expressed as g of the measured analyte (i.e. Gallic acid (GAE) or Fe^{3+}) per kilogram of fruit. Oxidative stress markers (H_2O_2 and MDA) were expressed as µmol kg^{-1} fruit. The data were analyzed using the general linear model procedure to determine differences among treatments and interaction effects using the statistical software JMP (version 11 SAS Institute Inc., NC, USA). All data were verified for normal distribution and homoscedasticity of residues and accordingly, enzymatic activities and microbiological data were transformed to $\log_{10}$ of U mg^{-1} protein or CFU g^{-1}, respectively. Transformed results were schematically represented as back-transformed means ± standard error of the mean. Transformed and non-transformed means were compared by analysis of variance (ANOVA) and separated by Tukey's test ($P < 0.05$).

3 Results

3.1 Microbial dynamics

Initial populations of CPA-7 on the fruit were 5.53 ± 0.03 $\log_{10}$ (Fig. 1A). Low oxygen concentration associated to modified atmosphere packaging (MAP) slowed down CPA-7 growth if compared to the samples stored in air, delaying the start of the exponential phase from 1 d to 3 d post-inoculation. On day 6, samples stored in modified atmosphere packaging showed a 0.5 $\log_{10}$ reduction of CPA-7 growth in respect of the samples stored in aerobic conditions. Mesofils populations were initially the same (1.7 ± 0.2 $\log_{10}$) in all samples (Fig. 1B). However differences in growth (1 $\log_{10}$ CFU g^{-1} fruit) among aerobic and modified atmosphere-stored samples became evident on day 6.

3.2 Fruit physicochemical quality parameters

No significant differences were observed among values of L* (lightness) and a* (redness) in the presence of CPA-7 (Table 1). Likewise, no differences were observed in the content of soluble solids or pH in response to the antagonist under any of the tested conditions throughout storage. Firmness was better maintained throughout the

242 studied period in the presence of CPA-7 if compared to non-inoculated fruit, in which it
243 was reduced at the end of storage.

244 3.3 Biochemical analyses

245 *3.1.1 Total antioxidant capacity (TAC) and total phenolic content (TPC)*

246 At the beginning of the experiment total antioxidant capacity (TAC), measured by the
247 content of Fe^{3+}, was 13.6 ± 0.2 g kg^{-1} fruit in all samples and gradually decreased
248 during storage (Fig. 2A). However, the drop of TAC was significantly slower in
249 presence of CPA-7 in aerobic conditions where it remained at 12.3 ± 0.4 g kg^{-1} fruit
250 until day 6. No differences were observed among MAP-stored samples in the first three
251 days of the experiment. However, on day 6 in MAP conditions, it was recorded a
252 reduction by 0.8-fold in response to CPA-7 if compared to the control.

253 Total phenolic content (TPC) was initially 5.1 ± 0.1 g kg^{-1} fruit in all samples regardless
254 of the presence of the antagonist (Fig. 2B). On day 3 and for both storage conditions,
255 the inoculation with CPA-7 was associated to a significantly higher amount of TPC (4.5
256 ± 0.1 g kg^{-1} fruit), than in the control samples (4.2 ± 0.04 g kg^{-1} fruit in air and 3.7 ± 0.04
257 g kg^{-1} fruit in MAP). In agreement to TAC, on day 6, TPC was reduced in MAP-stored
258 samples in response to CPA-7 (4.1 ± 0.1 g kg^{-1} fruit) if compared to the control (4.9 ±
259 0.1 g kg^{-1} fruit).

260 *3.1.2 Hydrogen peroxide production and malondialdehyde content (MDA)*

261 No sign of peroxidation of the membranes could be estimated from the measurement
262 of the content of the marker malondialdehyde (0.019 ± 0.04 µmol kg^{-1} fruit) under any
263 of the conditions tested during the analyzed period (data not shown). When combined
264 to the antioxidant, CPA-7 was able to erase the oxidative burst or at least that referred
265 to H_2O_2 accumulation in the fruit stored in air (Fig. 3). Contrastingly, in the samples
266 stored in MAP H_2O_2 drastically increased in the first 24 h reaching up to 1.4-fold higher
267 in response to CPA-7 than in the non-inoculated control. In general, metabolization of

this molecule was significantly slower in the MAP-stored samples in the presence of CPA-7 than in the rest of the samples. In this sense, values remained 13-fold higher if compared to the control at the end of storage. Contrastingly, in inoculated samples stored in air, the levels of H_2O_2 remained close to zero throughout the whole experiment.

3.1.3 Activities of enzymes involved in antioxidant metabolism

Changes in the enzymatic antioxidant potential of 'Golden delicious' fresh-cut apple differed throughout storage according to the inoculation with the antagonist and/or to the storage conditions. SOD activity decreased from 5554 ± 42 U mg^{-1} protein at initial time to 4863 ± 111 U mg^{-1} protein after 24 h in all samples, except for the CPA-7-inoculated MAP-stored sample, in which it remained stable up to day 3. This contrasted with the drop (0.7-fold lower than the CPA-7-inoculated sample) observed for the correspondent control (Fig 4A). In contrast, on day 6, SOD activity was oppositely modulated by CPA-7 depending on the storage conditions; it was 0.7-fold lower than the control when stored in MAP and 0.6-fold higher than the control when stored in air.

Catalase activity immediately after processing was similar in all samples (237 ± 17 U mg^{-1} protein) regardless of any of the factors assayed (Fig. 4B). However, it differentially changed in response to the antagonist in different storage conditions. It increased by 0.7-fold compared to the initial value when stored in air, reaching the same level than the control after 24 h. In contrast, CAT activity in inoculated samples was inhibited in response to the antagonist when stored in MAP (by more than 0.6-fold if compared to the control) during the first 3 d of storage.

Initially, PPO activity was 243 ± 7 U mg^{-1} protein which was reduced by 0.5-fold in the first 24 h (Fig. 4C). Thereafter, it drastically increased in response to CPA-7 in MAP conditions reaching almost 8-fold the activity showed by the correspondent non-inoculated fruit. However, when stored in air, CPA-7 had no significant effect in PPO activity throughout the analyzed period.

Activity of APX was initially around 348 ± 43 U mg^{-1} protein in all samples (Fig 4D). During the first 24 h post-inoculation, APX activity was induced up to 3-fold, if compared to the initial values, in response to the antagonist when stored in air. At this sampling time, peaks of APX activity in response to CPA-7 triplicated the activities recorded for the controls, regardless of the storage conditions. An enhanced APX activity associated to the antagonist was maintained until day 3 (1.8 fold higher than the control) in air-stored samples.

Immediately after inoculation, POX activity was inhibited in response to CPA-7 (by 0.6-fold if compared with the control) (Fig. 4E). Thereafter, this enzyme showed a biphasic activation in response to CPA-7 in aerobic conditions reaching values 1.5-fold and 1.7-fold higher than those observed for the control after 1 and 6 d post-inoculation, respectively. In contrast, POX activity was reduced in all MAP-stored samples during the first 24 h post-inoculation, but it attained significantly lower values (98 ± 10 U mg^{-1} protein) in the presence of the antagonist than in the control (84 ± 5 U mg^{-1} protein). As for air-stored samples on day six, POX activity was enhanced in the CPA-7-inoculated MAP-stored samples, reaching values eight fold higher than the control.

4 Discussion

Processing of fresh produce implies the removal or damage of most of the constitutive barriers of plants and generally activates inducible defense mechanisms to protect themselves against pathogens (Reviewed by Hodges and Toivonen, 2008). Pseudomonads that have been used as biocontrol agents (BCA) have shown to enhance plant response including cell-wall straightening, oxidative burst and expression of defense related genes that are usually activated by pathogens (Van der Ent et al., 2009). This phenomenon ‘primes’ plant metabolism allowing it to react faster and stronger upon a subsequent pathogen attack (Pieterse et al., 2001). One of the enzymes associated to resistance responses is peroxidase (POX) which mediates the last steps of the biosynthesis of lignin and hydrogen peroxide (H_2O_2) (Hiraga, 2001).

322 POX also catalyzes single-electron oxidation of phenolic compounds in the presence of
323 hydrogen peroxide. This latter molecule is one of the first molecules generated in plant
324 reaction to wounding and pathogen infection and has been postulated to play a dual
325 role as signaling factor to induce defense genes related to wound-stress and
326 hypersensitive response and it is also involved in fine tune regulation of oxidative
327 stress caused by reactive oxygen species (ROS) both locally and systemically (Hung et
328 al., 2005; Sewelam et al., 2014; Baxter et al., 2014). However, little is known about the
329 oxidative response in fresh-cut fruit associated to BCA.

330 The results obtained in the present study showed that oxidative response of 'Golden
331 delicious' fresh-cut apples was differentially modified in the presence of CPA-7
332 depending on the storage conditions. For instance, immediately after processing and
333 treatment with the antioxidant, H_2O_2 quickly began to accumulate in all samples to up to
334 the first 24 h in the MAP-stored samples and up to 3 d in the air-stored control. Similar
335 H_2O_2 accumulation pattern has been previously observed in non-inoculated fresh-cut
336 'Fuji' apples treated with ascorbic acid and stored in aerobic conditions (Larrigaudiere
337 et al., 2008). However, in the presence of CPA-7, the oxidative burst was practically
338 erased in the air-stored samples, and H_2O_2 levels were kept close to zero during the
339 whole experiment. Activation of ROS-scavenging enzymes correlated with the initial
340 oxidative burst associated with processing, thus the levels of SOD activity were initially
341 elevated in all samples. Subsequently, SOD activity decreased in all samples and APX
342 was sharply and transiently induced by CPA-7 as a first line of defense against
343 elevated H_2O_2 production in both MAP and air-stored samples after the first 24 h. On
344 the other hand, when stored in air CPA-7 was also associated to an early increase of
345 CAT and POX activities which could contribute to quickly eliminate H_2O_2 as soon as it
346 was produced. The decline of SOD activity 24 h after processing was also observed by
347 Larrigaudiere et al. (2008) in antioxidant-treated Fuji apples.

In the conditions assayed herein, as time passed, stress associated to mechanical damage was added to that caused by low-oxygen atmosphere as in MAP-stored samples, hence leading to changes in the modulation of some scavenging enzymes aiming to maintain redox homeostasis. In this way, from day 1 to 3, SOD activity peaked in the CPA-7-inoculated MAP-stored samples and subsequently, PPO and POX were sharply induced until the end of storage counteracting the inhibition of CAT. In contrast, in the air-stored samples the activation of SOD due to CPA-7 lasted until day 6 and was paired to the induction of POX. Thus, stressful conditions were associated not only to increased ROS-scavenging enzymes activities but to changes in their balance. It has been previously observed that reduced CAT activity is sometimes compensated by induction of APX and glutathione peroxidase (GPX) (Apel and Hirt, 2004). Similarly, Wang et al. (2014) observed that the treatment of loquat fruit with the antagonist *Bacillus cereus* AR-156 induced SOD activity while reduced the activities of CAT and APX enhancing the plant response to infection by *Colletotricum acutatum*.

The modulation of oxidative metabolism in fruit and vegetable in response to *Pseudomonas* spp. as observed in the present study, has been previously reported and correlated to the induction of plant resistance to several plant pathogens (Sangeetha et al., 2010). For instance, combinations of a non-fluorescent *Pseudomonas* spp. strain NFP6, *P. fluorescens* and *B. subtilis* induced up to 4-fold PAL, POX and PPO activities and increased by 3.6-fold the phenolic content in banana fruits challenged with crown rot pathogens, 5 d after treatment with the antagonists (Sangeetha et al., 2010). Likewise, *Pseudomonas corrugate* strain 13 and *Pseudomonas aerofasciens* strain 63-28 significantly induced PAL, POX and PPO in cucumber 2-5 d following treatment and elicited the defense response to subsequent infection with *Phytium aphanidermatum* (Chen et al., 2000). Similarly, mixtures of fluorescent rhizosphere and endophytic Pseudomonads induced these enzymes when sprayed on banana plants which subsequently showed enhanced systemic resistance

375 against bunchy top virus (Harish et al., 2009). Similar response regarding POX, PPO
376 and SOD activities was previously observed for other biocontrol agents such as
377 *Bacillus amyloliquefaciens* LJ02 after being sprayed on three leave-stage seedlings (Li
378 et al., 2015). In general accordance with our study, they observed a 2-fold increase of
379 POX activity on day 6 as well as an enhanced SOD activity in aerobic conditions until
380 day 5 priming plants for resistance to powdery mildew caused by the fungus
381 *Spaheroteca fugilinea.*

382 Changes in PPO activity in response to CPA-7 has been previously investigated in
383 fresh-cut 'Piel de sapo' melon stored in air and in MAP conditions during 8 d of storage
384 at 5 ºC (Plaza et al., 2016). In contrast to our results, no variation was recorded in
385 response to CPA-7 for MAP-stored samples. Furthermore, although an induction of this
386 enzyme associated to the antagonist was observed by Plaza et al. (2016) after 8 d of
387 storage in aerobic conditions compared to the initial activity, this increase did not differ
388 from the control. Differences in sampling days between that study and ours could
389 suggest that PPO could peak after 6 d of MAP-storage and drop before day 8 and in
390 the same way, the increase of PPO activity in aerobic samples could be produced after
391 6 d of storage. Alternatively, discrepancies between both studies could be associated
392 to the differential behavior of the bacterium in relation to different fruit matrices.

393 The question still remains on whether the increase of ROS-scavenging enzymes
394 activities such as superoxide dismutase, catalase and peroxidase is only a plant
395 response or is also a strategy deployed by the antagonist. This would confer this
396 bacterium with the ability to tolerate highly oxidant conditions generated after
397 processing of fresh produce and could act as a pivotal mechanism of action to
398 outcompete foodborne pathogens or spoilage microorganisms. Differences in the
399 modulation pattern of the oxidative metabolism throughout storage observed in the
400 present study did not correspond to great changes in CPA-7 populations as a function
401 of storage conditions. The tracking of population dynamics showed that CPA-7 was

able to tolerate the changes produced in the fruit oxidative metabolism during the assayed period. In this sense, CPA-7 showed similar growth upon refrigerated conditions both in air and in MAP with a slight inhibition corresponding to lower oxygen availability after 6 d of incubation. Previous semi-commercial trials performed with fresh-cut apples in conditions resembling those assayed herein showed similar CPA-7 population dynamics and revealed an inhibitory or bacteriostatic effect of this antagonist on *L. monocytogenes* depending on the temperature of storage rather than on MAP or air storage conditions (Alegre et al., 2013a). From this perspective, high resistance to ROS of the antagonistics yeasts (*Cryptococcus laurentii* LS-28 and *Rhodotorula glutinis* LS-11) inoculated in wounded apple and peach fruits has been associated with enhanced antagonistic activity against *Botrytis cinerea* and *Penicilium expansum* (Castoria et al., 2003*;* Zhang et al., 2017).

On the other hand, concomitant with enzymatic changes, increased non-enzymatic antioxidant activity provides host tissue with additional defense tools to counteract ROS production induced by both biotic and abiotic stress (Hung et al., 2005). The initial accumulation of phenolic compounds, as observed in our trials regardless of the presence of the antagonist, has been studied for fresh-cut produce (Saxena et al., 2009). It could be related to the high production of H_2O_2 upon mechanical injury through an enhanced respiration or due to the activation of several metabolic pathways such as the hexose –monophosphate shunt pathway, the acetate pathway and/or the release of bound phenolic compounds by hydrolytic enzymes (Harish et al., 2009). However, the subsequent reduction of total phenolic content was slowed down in the presence of CPA-7 when stored in aerobic conditions at least until day 3 which could also account for the maintenance of beneficial nutritional properties of the fruit. Contrastingly, in MAP conditions, CPA-7 was associated to a peak in the production of phenolic compounds on day 3 with a concomitant increase of PPO activity. Interestingly, under such storage conditions, the drastic accumulation of this enzyme at

429 the end of the storage period was correlated to a significant drop of its substrates, yet it
430 did not correlate with a significant increase of browning.

431 Regarding the effect of the antagonist on fruit quality, CPA-7 had a positive effect on
432 the maintenance of physical parameters such as firmness throughout storage which
433 contrasted with control samples. This result agreed with the maintenance of the cellular
434 membranes integrity in the presence of CPA-7 as estimated chemically from the
435 measure of MDA. No significant effect on pH or soluble solids was observed due to the
436 antagonist during the studied period. The combination of the antagonist with the
437 antioxidant agent resulted in the maintenance of luminosity and redness but similar to
438 the results observed for the control samples at all times of analysis. Similarly, in CPA-
439 7-inoculated fresh-cut 'Golden delicious' apples treated with 60 g L^{-1} Natureseal®
440 (AS1), as in the present study, L* and a* Hunter values were maintained during 14 d of
441 storage at 5 ºC (Alegre et al., 2013a). The same antioxidant treatment has been
442 previously shown to efficiently maintain color properties (with a* values from -2.55 to -
443 3.14) and to increase the firmness of fresh-cut of apple wedges from ten cultivars after
444 storage for 5 d at 2 ºC – 4 ºC (Roble et al., 2009). In contrast, when using a different
445 antioxidant treatment (5 g L^{-1} calcium chloride), a decrease of luminosity of CPA-7-
446 treated fresh-cut melon after 8 d of storage at 5 ºC was observed by Plaza et al.
447 (2016).

448 ## 5 Conclusions

449 The results showed herein indicated that CPA-7 was associated to a differential
450 modulation of the oxidative response of antioxidant-treated fresh-cut apple during
451 chilling storage in MAP or aerobic conditions. CPA-7-inoculated fruit showed a higher
452 antioxidant capacity, a higher accumulation of phenolic compounds and an enhanced
453 activity of key antioxidant enzymes (POX, SOD and APX) which were related to the
454 plant response to either mechanical injury or microbial invasion, at certain moments of
455 storage. The antagonist ability to tolerate or to mediate these changes and to grow

upon such conditions could imply high resistance to oxidative stress which would, in turn, provide CPA-7 with an adaptive advantage to outcompete putative pathogen infection. The differential temporal enhancement of the analyzed enzymes depending on the storage conditions suggests a synergistic activation of different pathways in response to the several factors associated to production and commercialization of fresh-cut fruit such as mechanical injury, low-temperature, antioxidant-treatment and low-oxygen conditions. Further studies including transductional analysis of the accumulation patterns of different antioxidant isoenzymes, which may be activated by the plant and/or the antagonist upon challenge with foodborne pathogens would improve the understanding of such mechanisms.

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620 Table 1. Quality parameters of fresh-cut antioxidant-treated 'Golden delicious' apple
621 wedges inoculated with *P. graminis* CPA-7 (CPA) or non-inoculated (NI) during storage
622 in aerobic conditions (AIR) or in modified atmosphere packaging (MAP).

Parameter	Treatment	Time (days) at 5 ºC											
		0			1			3			6		
Lightness (L*)	**AIR NI**	78.1 ± 0.5	a	B	80.9 ± 0.2	a	A	79.7 ± 0.4	a	AB	78.5 ±0.6	abc	B
	AIR CPA	78.5 ± 0.9	a	B	77.5 ± 0.4	bc	B	78.8 ± 0.6	ab	B	78.5 ±0.7	abc	B
	MAP NI				78.2 ± 0.6	bc	A	79.8 ± 0.5	a	A	79.1 ±0.5	ab	A
	MAP CPA				80.1 ± 0.3	ab	A	79.0 ± 0.5	ab	A	79.7 ±0.4	a	A
Redness (a*)	**AIR NI**	-6.6 ± 0.4	b	B	-6.4 ± 0.4	a	A	-6.4 ± 0.3	cd	A	-5.8 ±0.5	bc	A
	AIR CPA	-6.1 ± 0.6	ab	B	-6.0 ± 0.3	bc	B	-6.5 ± 0.2	ab	B	-6.2 ±0.4	bc	B
	MAP NI				-6.4 ± 0.5	bc	AB	-7.7 ± 0.1	d	B	-6.4 ±0.1	bc	A
	MAP CPA				-7.2 ± 0.5	ab	A	-6.0 ± 0.3	cd	A	-6.5 ±0.3	c	A
Firmness (N)	**AIR NI**	13.1 ± 1.0	a	A	12.1 ± 0.8	a	A	12.0 ± 0.9	a	A	11.5 ±0.6	a	B
	AIR CPA	13.0 ± 0.8	a	A	12.8 ± 0.8	a	A	12.5 ± 0.6	a	A	13.0 ±0.6	a	A
	MAP NI				14.3 ± 0.2	a	A	12.2 ± 0.6	a	AB	11.7 ±0.3	a	B
	MAP CPA				11.3 ± 1.5	a	A	11.7 ± 0.5	a	A	13.1 ±0.9	a	A
pH	**AIR NI**	3.8 ± 0.1	a	A	4.0 ± 0.1	a	A	3.9 ± 0.02	a	A	3.7 ±0.1	a	A
	AIR CPA	4.1 ± 0.1	a	A	3.9 ± 0.1	a	A	4.1 ± 0.1	a	A	3.9 ±0.2	a	A
	MAP NI				3.85 ± 0.03	a	A	3.9 ± 0.1	a	A	3.9 ±0.1	a	A
	MAP CPA				3.9 ± 0.1	a	A	3.9 ± 0.1	a	A	4.15 ±0.04	a	A
Titratable acidity as malic acid content (g L^{-1} juice)	**AIR NI**	3.3 ± 0.2	a	A							3.8 ±0.3	a	A
	AIR CPA	3.22 ± 0.02	a	A							3.6 ±0.5	a	A
	MAP NI										3.5 ±0.3	a	A
	MAP CPA										3.6 ±0.2	a	A
Soluble solids (%)	**AIR NI**	12.93 ± 0.04	a	A							13.3 ±0.9	a	A
	AIR CPA	12.80 ± 0.06	a	A							12.6 ±0.1	a	A
	MAP NI										13.3 ±0.1	a	A
	MAP CPA										12.6 ±0.3	a	A

Values are means ± standard error of the mean. Different lowercase letters represent significant differences among treatments at each sampling time. Different capital letters represent differences for the same treatment throughout time according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$).

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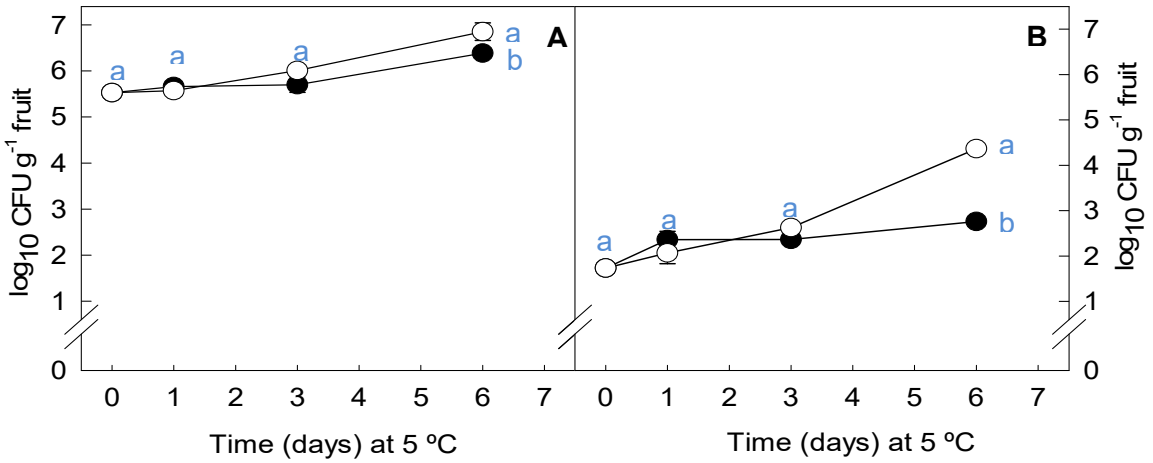

630 Figure 1. Population dynamics of (A) *P. graminis* CPA-7 and (B) total mesofils on
631 'Golden delicious' fresh-cut apple wedges treated with antioxidant, during six days of
632 storage at 5 ºC in modified atmosphere packaging (MAP) (●) or in air (○). Each point
633 represents the mean and error bars represent the standard error of the mean (n = 6).
634 Different letters represent significant differences among treatments at each sampling
635 point according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$).
636 Underlined letters represent equal means that correspond with overlapped symbols in
637 the graph.

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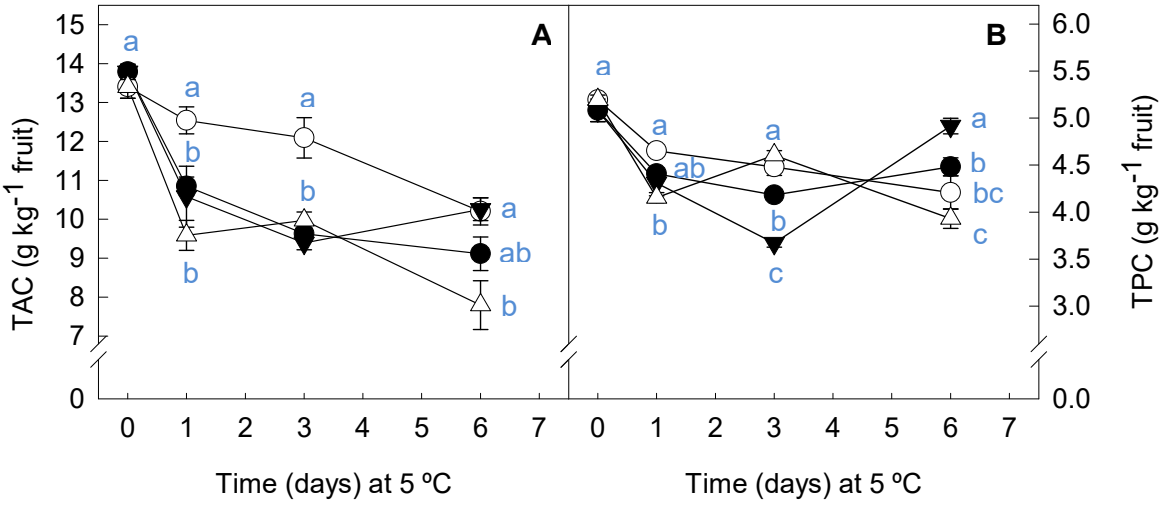

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641 Figure 2. Changes in the levels of (A) Total Antioxidant Capacity, TAC, estimated from
642 the amount of Fe^{3+} and (B) Total Phenolic Content, TPC, estimated from the amount of
643 Gallic acid, in fresh-cut 'Golden delicious' apple wedges treated with antioxidant after
644 inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated
645 control fruit during storage in air (●) or in MAP (▼). Each point represents the mean
646 and error bars represent the standard error of the mean (n = 9). Different letters
647 represent significant differences among treatments at each sampling point according to
648 analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters
649 represent equal means that correspond with overlapped symbols in the graph.

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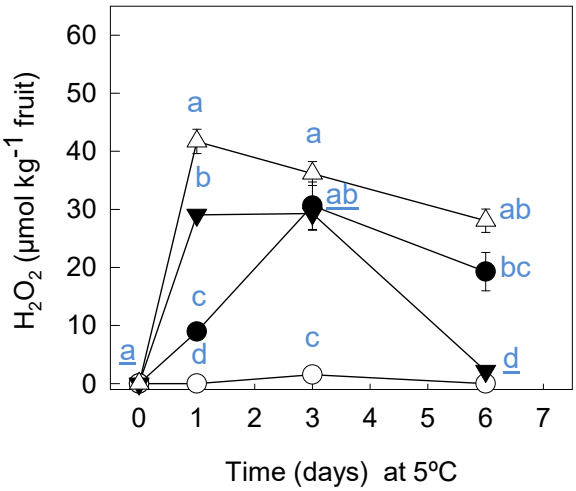

Figure 3. Changes in the levels of hydrogen peroxide (H_2O_2) in 'Golden delicious' fresh-cut apple wedges treated with antioxidant after inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in MAP (▼). Each point represents the mean and error bars represent the standard error of the mean (n = 9). Different letters represent significant differences among treatments at each sampling point according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters represent equal means that correspond with overlapped symbols in the graph.

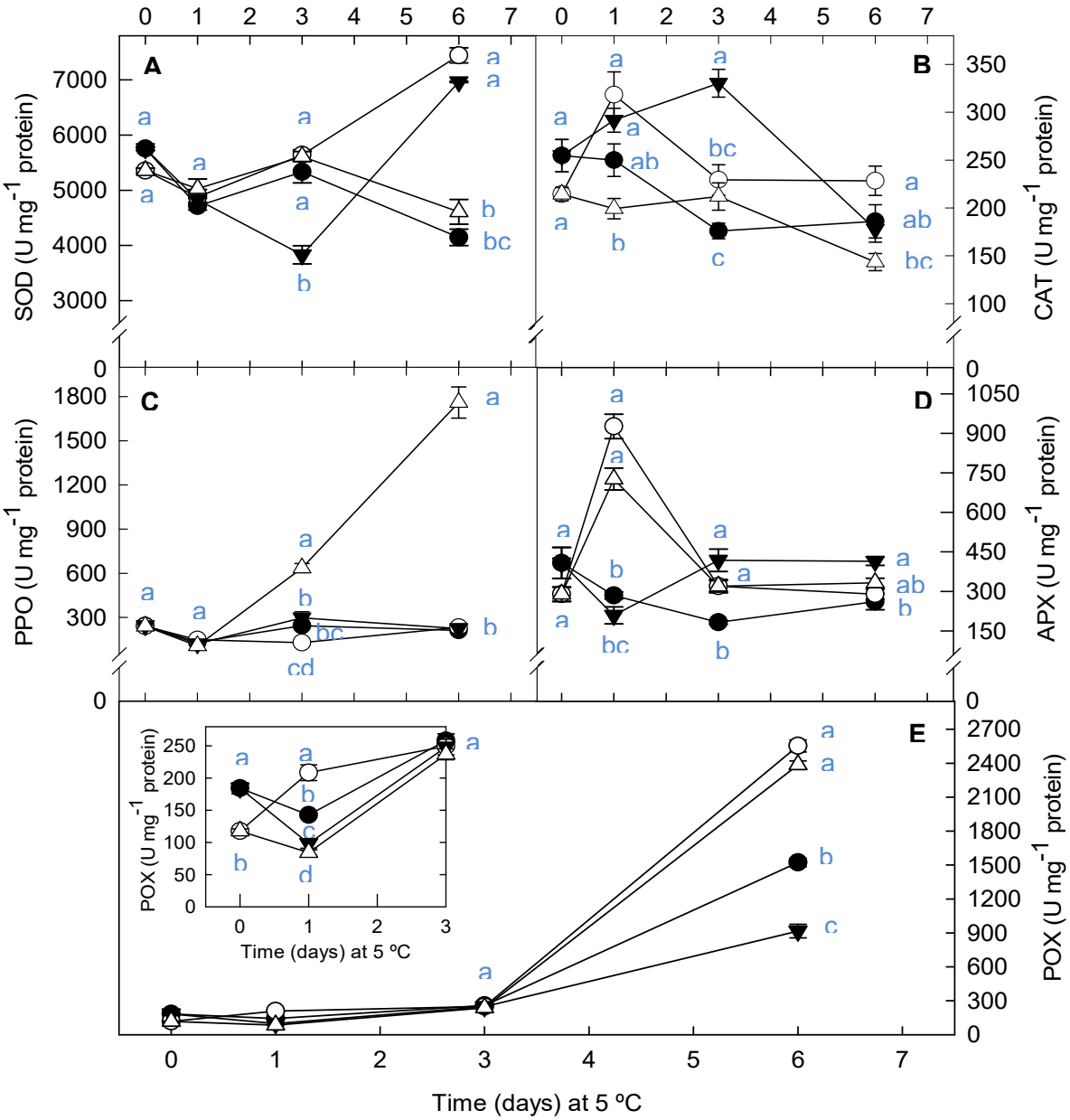

Figure 4. Changes in the activity of the enzymes A) superoxide dismutase (SOD), B) catalase (CAT), C) polyphenol oxidase (PPO), D) ascorbate peroxidase (APX) and E) peroxidase (POX) in 'Golden delicious' fresh-cut antioxidant-treated apple wedges, after inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in MAP (▼). The internal plot in graph E represents the first three sampling times at a smaller scale. Each point represents the mean and error bars represent the standard error of the mean (n = 9). Different letters represent significant differences among treatments at each sampling

673 point according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$).
674 Underlined letters represent equal means that correspond with overlapped symbols in
675 the graph.

676

677